

Interaction Between Birthweight and Polymorphism in the Calcium-Sensing Receptor Gene in Determination of Adult Bone Mass: The Hertfordshire Cohort Study

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ABSTRACT. Objective. We sought evidence of interaction between single-nucleotide polymorphisms (SNP) in the calcium-sensing receptor (*CASR*) gene and early life in determination of bone mineral density (BMD) among individuals from the Hertfordshire Cohort Study.

Methods. Four hundred ninety-eight men and 468 women aged 59–71 years were recruited. A lifestyle questionnaire was administered and BMD at lumbar spine and femoral neck was measured. DNA was obtained from whole blood samples using standard extraction techniques. Five SNP of the *CASR* gene termed CASRV1 (rs1801725, G→T, S986A), CASRV2 (rs7614486, T→G, untranslated), CASRV3 (rs4300957, untranslated), CASRV4 (rs3804592 G→A, intron), and CASRV5 (rs1393189, T→C, intron) were analyzed.

Results. Among women the 11 genotype of the CASRV3 SNP was associated with higher lumbar spine BMD within the lowest birthweight tertile, while the opposite pattern was observed among individuals in the highest birthweight tertile (test for interaction on 1 df, $p = 0.005$, adjusted for age, body mass index, physical activity, dietary calcium intake, cigarette and alcohol consumption, social class, menopausal status, and hormone replacement therapy use). Similar relationships were seen at the total femur ($p = 0.042$, fully adjusted) with birthweight and at the total femur according to weight at 1 year tertile among women ($p < 0.001$, fully adjusted). One haplotype was associated with lumbar spine BMD in women ($p = 0.008$, fully adjusted); these findings were replicated in a second cohort.

Conclusion. We have found evidence of an interaction between a SNP of the *CASR* gene and birthweight in determination of bone mass in a UK female population. (First Release Feb 15 2007; *J Rheumatol* 2007;34:769–75)

Key Indexing Terms:

BONE BONE DENSITY COHORT STUDIES GENETIC STUDIES

Twin and family studies confirm an inherited contribution to peak bone mass, and various candidate genes have been proposed for the genetic regulation of bone mineral, including the genes for the vitamin D receptor, the estrogen receptor, and type I collagen (Col IA1). However, polymorphisms in these genetic loci explain only a small portion of the observed variance in bone mass in the general population¹⁻⁵. Evidence is

also accumulating that the risk of later osteoporosis might be programmed by environmental influences during intrauterine or early postnatal life. Growth in infancy, a marker of such programming, predicts adult bone mass independently of adult lifestyle⁶⁻⁹. We published evidence of such an interaction between polymorphisms of the growth hormone gene, birthweight, and adult bone mass in a cohort of Hertfordshire, UK, men and women^{10,11}. However, little is known of the interaction between intrauterine environment and genetic markers of calcium (Ca) metabolism and their association with adult bone mineral density (BMD).

In vitro studies have demonstrated that chondrogenic and osteogenic function can be influenced in response to different extracellular Ca concentrations¹². Heath, *et al* described a polymorphism in exon 7 of the Ca-sensing receptor (*CASR*) gene, coding the intracellular domain of the receptor¹³. This polymorphism, A986S, was found to be a predictor of circulating Ca concentrations in women^{14,15}. However, to date, results relating this gene to BMD have been conflicting. We sought to evaluate the interaction between polymorphisms of the *CASR* gene and early life in the determination of BMD in a large well-characterized elderly cohort.

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Supported by grants from the the Arthritis Research Campaign and the Medical Research Council.

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Accepted for publication November 29, 2006.

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MATERIALS AND METHODS

In our study, which was designed to examine the relationship between growth in infancy and the subsequent risk of osteoporosis, the selection procedure was as follows: in brief, with the help of the National Health Service Central Registry at Southport and Hertfordshire Family Health Service Association, we traced men and women born between 1931 and 1939 in Hertfordshire, who still lived in East Hertfordshire in 1998. With written permission from each subject's general practitioner, we approached each person by letter, asking if he or she would be willing to be contacted by one of our research nurses. If the subjects agreed, a research nurse performed a home visit and administered a structured questionnaire. This included information on socioeconomic status, medical history, drug history, cigarette smoking, alcohol consumption, and reproductive variables in women. A total of 768 men and 714 women completed the home visit; of this number, 737 men and 675 women subsequently attended clinic.

At clinic, height was measured to the nearest 0.1 cm using a Harpenden pocket stadiometer (Chasmors Ltd., London, UK) and weight to the nearest 0.1 kg on a Seca floor scale (Chasmors Ltd.). Venous whole blood samples were taken at this clinic visit.

Eligible subjects were then invited to reattend for bone density measurements. Individuals taking drugs known to alter bone metabolism (such as bisphosphonates) were excluded from this part of the study, although women taking hormone replacement therapy (HRT) were allowed to participate. There were no other exclusion criteria. BMD was measured in 498 men and 468 women, by dual energy radiographic absorptiometry at the lumbar spine and proximal femur (neck, total, intertrochanteric and trochanteric regions, Ward's triangle) using a Hologic QDR 4500 instrument (Vertec Scientific, Reading, UK). Measurement precision error, expressed as coefficient of variation, was 1.55% for lumbar spine BMD, 1.45% for total femur, and 1.83% for femoral neck BMD for the Hologic QDR 4500; these figures were scans on the same day, getting on and off the table between examinations. Short-term (2 mo) precision error for the QDR 4500 was less than 1% for both sites (manufacturer's figures).

Genomic DNA was extracted from whole blood samples according to standard procedures. Single-nucleotide variants in the *CASR* gene termed CASRV1 (rs1801725, G→T, S986A), CASRV2 (rs7614486, T→G, untranslated), CASRV3 (rs4300957, untranslated), CASRV4 (rs3804592 G→A, intron), and CASRV5 (rs1393189, T→C, intron) were analyzed.

The different alleles were termed 1 or 2, resulting in genotypes 11, 12, and 22. Genotypes 12 and 22 were combined in the event of a low frequency of genotype 22. Analyses were conducted separately for men and women using Stata 8. The relationship between each continuously distributed phenotype variable and each single-nucleotide polymorphism (SNP) was explored using both analysis of variance and linear regression models. Analyses were conducted with and without adjustment for age, body mass index (BMI), typical activity level, dietary calcium intake, smoking status, alcohol intake, current social class, and menopausal status and HRT use for women. Haplotypes were inferred using the Phase package¹⁶.

The East and North Hertfordshire Ethical Committees granted ethical permission for the study. All participants gave written informed consent.

RESULTS

The characteristics of the study population at baseline are displayed in Table 1. The mean age of the men was 64.3 and the women 65.6 years. Thirty-four percent of the men and 62% of women had never smoked, while 52% of men (28% of women) were ex-smokers and 15% of men (10% of women) were current smokers. Four percent of men and 18% of women were non-drinkers, while 21% of men and 12% of women were moderate drinkers (11–21 and 8–14 units of alcohol/wk, respectively, 1 unit being a single glass of wine or a single measure of spirits).

Table 1. Summary characteristics of study participants. Data are mean (SD) unless stated otherwise.

Characteristic	Men (n = 498)	Women (n = 468)
Age, yrs	64.3 (2.5)	65.6 (2.5)
BMI, kg/m ² *	26.6 (1.1)	26.8 (1.2)
Alcohol consumption (units per week)**	10.0 (3.0, 22.5)	2.5 (0.5, 7.0)
Habitual activity, % ***	64.0 (14.8)	61.3 (14.9)
No. (%), current manual social class (I-III-V) [†]	277 (55.6)	286 (61.1)
No. (%), current non-manual social class (I-II-III) [†]	193 (38.8)	182 (38.9)
Lumbar spine BMD, g/cm ² ††	1.08 (0.16)	0.96 (0.17)
Femoral neck BMD, g/cm ² ††	0.85 (0.12)	0.76 (0.12)
Total femoral BMD, g/cm ² ††	1.04 (0.13)	0.90 (0.13)

* Geometric mean (SD). ** Median and interquartile range among drinkers; 20 men and 86 women stated they do not drink alcohol. *** Standardized score range 0–100 derived from frequency of gardening, housework, climbing stairs, and carrying loads in a typical week. Higher scores indicate greater level of activity. [†] Social class was unclassified for 28 men. I-III and III-V denote classes 1–3 (non-manual), and 3 (manual) to 5, of the 1990 OPCS Standard Occupational Classification scheme for occupation and social class. Social class was identified on the basis of own current or most recent full-time occupation for men and never-married women, but on the basis of the husband's occupation for ever-married women. ^{††} 1 man was not scanned at the lumbar spine; 3 men and 1 woman were not scanned at the hip. BMI: body mass index; BMD: bone mineral density.

The distribution frequencies of each genotype were as follows: CASRV1: 11 – 78.1%, 12/22 – 21.9%; CASRV2: 11 – 58%, 12 – 35.7%, 22 – 6.3%; CASRV3: 11 – 71.1%, 12 – 26.0%, 22 – 2.9%; CASRV4: 11 – 72.7%, 12 – 25.0%, 22 – 2.3%; CASRV5: 11 – 80.8%, 12/22 – 19.3%. Among men, the CASRV2 22 genotype was underrepresented, and the CASRV2 12 genotype overrepresented ($p = 0.008$); similarly, among men the CASRV3 22 genotype was underrepresented and the CASRV3 12 genotype overrepresented ($p = 0.02$). None of the other genotypes differed by sex. There was no evidence of departure from Hardy-Weinberg equilibrium. A total of 22.2% had at least one S allele of the A986S polymorphism (coded CASRV1). Taking into account the poor power of the recessive genotype (SS), the population for this gene was divided into 2 groups according to the presence or absence of the S allele.

There were no statistically significant direct associations between any of the CASR SNP and bone mass at the lumbar spine or femoral neck in the cohort as a whole, whether women taking HRT were excluded or not (Table 2). However, we also investigated the possible interaction between genotype and early environment as predictors of bone mass (Table 3). Among women, the 11 genotype of the CASRV3 SNP was associated with higher lumbar spine BMD within the lowest birthweight tertile, while this genotype was associated with lower lumbar spine BMD in the highest birthweight tertile (test for interaction $p = 0.005$, fully adjusted for age, social

Table 2. Lumbar spine, femoral neck, and total femoral BMD in relation to genotype.

Genotype	CASRV1				CASRV3					
	Men		Women		Men		Women			
	11	12/22	11	12/22	11	12	22	11	12	22
Lumbar spine BMD	1.07 (0.16)	1.09 (0.15)	0.96 (0.18)	0.95 (0.16)	1.08 (0.16)	1.06 (0.16)	1.05 (0.14)	0.96 (0.17)	0.94 (0.16)	1.05 (0.24)
n	376	110	356	96	338	136	8	322	104	19
p value adjusted	0.194		0.127		0.239		0.120		0.614	
unadjusted	0.226		0.506		0.174		0.614			
Femoral neck BMD	0.85 (0.12)	0.84 (0.13)	0.76 (0.12)	0.75 (0.11)	0.85 (0.12)	0.85 (0.13)	0.90 (0.12)	0.76 (0.12)	0.74 (0.12)	0.75 (0.15)
n	374	110	355	96	336	136	8	322	103	19
p value adjusted	0.598		0.083		0.530		0.889		0.209	
unadjusted	0.465		0.502		0.787		0.209			
Total femoral BMD	1.04 (0.13)	1.03 (0.14)	0.90 (0.13)	0.89 (0.13)	1.04 (0.13)	1.03 (0.14)	1.07 (0.13)	0.90 (0.13)	0.89 (0.12)	0.91 (0.17)
n	374	110	355	96	336	136	8	322	103	19
p value adjusted	0.538		0.593		0.625		0.890		0.159	
unadjusted	0.615		0.126		0.859		0.159			

Values are presented as means (SD). P values are given unadjusted on 1 df and adjusted for age at clinic, BMI, physical activity score, dietary calcium intake, smoking status, alcohol unit intake per week, current social class, years since menopause, HRT status. BMD: bone mineral density.

Table 3. Lumbar spine and total femoral BMD in women according to genotype and birthweight and weight at 1 year.

CASRV3	Lumbar Spine BMD			Total Femoral BMD		
	11	12	22	11	12	22
Birthweight (tertile)						
Low ≤ 3175 g	0.96 (0.18)	0.92 (0.16)	0.90 (0.13)	0.90 (0.14)	0.87 (0.12)	0.86 (0.09)
n	129	51	6	129	51	6
Middle 3175–3629 g	0.98 (0.17)	0.97 (0.17)	1.05 (0.29)	0.90 (0.14)	0.91 (0.13)	0.94 (0.23)
n	111	34	7	111	34	7
High > 3629 g	0.95 (0.17)	0.95 (0.16)	1.19 (0.20)	0.89 (0.12)	0.91 (0.10)	0.94 (0.14)
n	82	19	6	82	18	6
p for interaction unadjusted	0.009			0.046		
adjusted	0.005			0.042		
Weight at 1 year of age (tertile)						
Low ≤ 9185 g	0.95 (0.15)	0.94 (0.15)	0.90 (0.18)	0.90 (0.12)	0.87 (0.13)	0.85 (0.05)
n	95	48	3	95	48	3
Middle 9185–9979 g	0.98 (0.17)	0.89 (0.14)	1.08 (0.22)	0.91 (0.14)	0.87 (0.11)	0.91 (0.19)
n	100	26	10	100	26	10
High > 9979 g	0.96 (0.19)	0.98 (0.19)	1.08 (0.31)	0.88 (0.14)	0.93 (0.11)	0.95 (0.17)
n	127	30	6	127	29	6
p for interaction unadjusted	0.17			0.003		
adjusted	0.07			0.000		

Values are presented as means (SD). P values for interaction are given unadjusted and adjusted (on 1 df) for age at clinic, BMI, physical activity score, dietary calcium intake, smoking status, alcohol unit intake per week, current social class, years since menopause, HRT status. BMD: bone mineral density.

class, BMI, physical activity, Ca intake, cigarette and alcohol consumption, menopausal status, and HRT use). Similar relationships were observed at the total femur (test for interaction, fully adjusted $p = 0.042$). In addition, the 1 allele of the CASRV3 SNP was associated with higher total femoral BMD among women in the lowest weight at 1-year tertile (test for interaction, fully adjusted $p < 0.001$); in the highest weight at 1-year tertile the 1 allele was, again, associated with lower total femoral BMD (Figure 1). We expanded these analyses to utilize other cutpoints for early life measures (birth weight < 2500 g, 2500–3000 g, 3000–3500 g, 3500–4000 g, > 4000 g).

While these revised groupings made little difference to our results, further interpretation was difficult due to the small numbers of women in the lowest birthweight group (11: 11 subjects; 12: 10 subjects; 22: 0 subjects). No significant genotype–early environment interactions were observed in men.

Six haplotypes of the 5 CASR SNP accounted for 92.3% of all the haplotypes defined in the Hertfordshire Cohort Study dataset: 11111, 11121, 12211, 21111, 11112, 12111. Significant associations were observed between the 12211 haplotype (the third most frequent in this population) and lum-

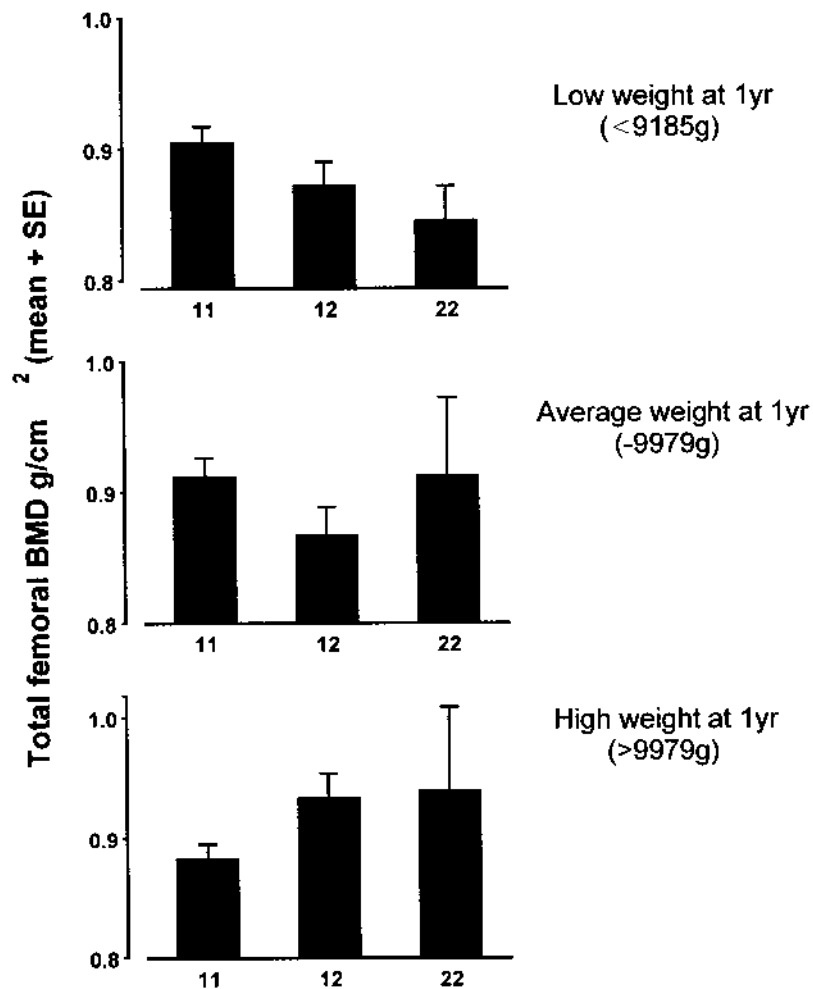


Figure 1. Total femoral BMD by CASRV3 genotype according to weight at 1 year of age in 444 women. See text for adjustments. $P < 0.001$ on 1 df.

Table 4. Associations of BMD with haplotypes of the CASR gene among men and women from the Hertfordshire Cohort Study.

Haplotype	Frequency (n)	Men			Frequency (n)	Women			
		Mean Lumbar Spine BMD, g/cm ²	Mean Femoral Neck BMD, g/cm ²	Mean Total Femoral BMD, g/cm ²		Mean Lumbar Spine BMD, g/cm ²	Mean Femoral Neck BMD, g/cm ²	Mean Total Femoral BMD, g/cm ²	
1	11111	0.43 (384)	1.05	0.85	1.04	0.46 (386)	0.97	0.76	0.90
2	11121	0.15 (129)	1.08	0.85	1.04	0.13 (108)	0.95	0.75	0.88
3	12211	0.15 (129)	1.03	0.85	1.03	0.11 (94)	1.00	0.76	0.91
4	21111	0.00 (0)	—	—	—	0.09 (76)	0.96	0.76	0.89
5	11112	0.11 (96)	1.07	0.84	1.04	0.06 (51)	0.96	0.79	0.92
6	12111	0.06 (55)	1.07	0.87	1.06	0.08 (70)	0.94	0.76	0.90
All	All	445 men	1.07 ± 0.01	0.85 ± 0.01	1.04 ± 0.01	423 women	0.96 ± 0.01	0.76 ± 0.01	0.90 ± 0.01

BMD: bone mineral density.

bar spine BMD in women (Table 4; $p = 0.008$, adjusted for age at clinic, BMI, physical activity, dietary Ca score, social class, cigarette and alcohol consumption, years since menopause and HRT use in women). We sought to replicate our findings using an older community from the Hertfordshire Cohort that

has been extensively described previously¹⁰. Indeed, our findings were replicated among this group of 186 men and 122 women aged 61–73 years at study, with the 12211 haplotype once again being associated with higher lumbar spine BMD among women ($p = 0.05$, fully adjusted).

DISCUSSION

We have demonstrated evidence of an interaction between a SNP of the *CASR* gene and early environment in the effect on BMD in a Hertfordshire population of healthy elderly women. To our knowledge, this is the first report relating *CASR* genotype to BMD taking into account the possible effects of development in early life. We have also demonstrated that one haplotype was associated with lumbar spine BMD in women, a finding replicated in a second population.

There were a number of limitations to this study. The individuals studied were selected because we have accurate records of their early life; these subjects were all born in Hertfordshire, and still live there. They have, however, previously been shown to have anthropometric and lifestyle characteristics similar to those of the general population¹⁷. The allelic frequency of the recessive alleles of both *CASRV1* and *CASRV3* polymorphisms was fairly low. However, the effect of the *CASRV3* recessive genotype is consistent in BMD measurements at different sites and different ages, suggesting that our results are not a coincidental finding. Our genotype frequencies of the A986S (*CASRV1*) polymorphism, however, are comparable to those found by other groups.

We included SNP located in noncoding and intergenic regions, rather than focusing exclusively on the coding region. It is hypothesized that variations underlying complex diseases are not limited to the structure of the encoded protein. Gene regulation is the result of the combinatorial action of multiple transcription factors binding at multiple sites in and near a gene and it has recently been shown that gene expression regulatory elements reside in noncoding and intergenic regions¹⁸.

Ca homeostasis has an important role in the regulation of bone remodeling, and alterations of the mechanisms involved in this regulation may contribute to the development of metabolic bone diseases. The main homeostatic regulator of extracellular Ca is the calcitropic parathyroid hormone (PTH). However, the *CASR* mediates this pathway through its ability to sense small changes in circulating Ca concentration and to activate intracellular pathways if its setpoint is not met. These pathways lead to an increase in PTH, which in turn will enhance the activation of vitamin D¹⁹. Both hormones increase circulating Ca by stimulating Ca resorption in renal tubules and intestine, and by stimulating bone resorption through promoting the differentiation of osteoclasts from multinucleated precursors^{20,21}.

The *CASR* is defective in individuals with familial hypocalciuric hypercalcemia (FHH) and neonatal severe hyperparathyroidism due to inactivating mutations^{22,23}. Heath, *et al* suggested that the NH₂-terminal extracellular and membrane-spanning regions of the receptor protein are functional domains for Ca binding and signal transduction, and mutations in these regions lead to familial benign hypocalciuric hypercalcemia¹³.

An association between a Ca-repeat polymorphism of the *CASR* gene and BMD was found by Tsukamoto, *et al*²⁴. Cole,

et al^{14,15} reported associations between the A986S polymorphism and Ca levels in a clinical trial of 163 White Canadian women. The AA genotype was associated with lower Ca levels, indicating a loss of function at the receptor level as in FHH. They suggested that this polymorphism would have a potential role as a predictor of disorders that affect bone and mineral metabolism. Lorentzon, *et al*²⁵ and Eckstein, *et al*²⁶ confirmed this effect of polymorphisms on circulating Ca concentrations and found associations with BMD. However, after correction for physical activity, the polymorphisms no longer had an effect on BMD. Eckstein, *et al* found the S allele to be overrepresented in a group of 80 Israelis with low BMD, although age at menarche was found to be the main predictor of BMD in their group. Several other groups have examined the relationship between extracellular Ca or BMD and the A986S polymorphism without finding a significant association. Takacs, *et al*²⁷ and Cetani, *et al*²⁸ studied the polymorphism in a homogenous postmenopausal White Hungarian and Italian, respectively, female population and confirmed the functionality of the polymorphism in circulating Ca levels, but could not confirm any influence of the polymorphism on BMD. Young, *et al*²⁹ and Bollerslev, *et al*³⁰ analyzed Ca data for, respectively, 102 New Zealand and 1252 Canadian postmenopausal women. Bollerslev, *et al* found no significance of the A986S polymorphism in association with serum Ca levels or with BMD. Most recently, Perez-Castrillon, *et al* failed to find an association of the A986S polymorphism with lumbar spine BMD in 48 hypertensive women³¹.

In our large homogeneous study group we found the same allele frequencies of the A and S alleles as previously found by other groups. However, we could not find a direct effect of the *CASR* A986S polymorphism on BMD, and can confirm that the results of the latest studies also apply for our UK cohort. There are several possible explanations for this negative result. The *CASR* may not have a crucial role in the regulation of osteoblast function. However, we also studied 4 other polymorphisms and found an interaction between the *CASRV3* (C→T, untranslated) polymorphism and development in early life in determination of BMD in women. We previously reported data suggesting that the intrauterine environment, using birthweight as a marker, and early life, using weight at 1 year of age as a marker, may modulate the relationship between the vitamin D receptor and growth hormone genes and adult BMD^{10,11,32}. Therefore we hypothesized that the effect of polymorphisms of the *CASR* gene on BMD may be modulated by development in early life. Our study provides evidence of an interaction between the early environment and a genotypic locus such that individuals who carry the unfavorable, recessive genotype who also grew poorly in fetal life and infancy are susceptible to lower BMD, whereas the recessive genotype predisposes to higher BMD among individuals that grew better in early life.

Men have greater bone mineral content and area, indicating a larger skeletal size, and this is normally reflected in a

higher BMD in men compared with women³³. However, when adequate correction for body size is performed (by calculation of bone mineral apparent density, for example), the apparent differences between the sexes are much reduced, or removed. However, there are also known to be sex differences in age-related changes in bone loss rates and bone strength. Hence, in one study, men had approximately 30% larger cross-sectional bone size compared with women at age 67–69 years³⁴. At all sites, women had 2- to 5-fold reduction in bone mass with age compared to men, but had comparable increments in bone size. This was reflected in significantly worse bone strength measures with age in women. The sex hormones are likely to be important contributors to the sexual dimorphism we describe. The effects of the sex steroid receptors on skeletal regulation have been tested recently in murine models³⁵; androgen receptor-deficient mice follow the male pattern of long bone development, but imitate females in bone density and trabecular bone, while loss of the α subtype of the estrogen receptor resulted in increased bone length in females but reduced bone length in males. Loss of either receptor resulted in increased osteoblast sensitivity to PTH. Our study suggested an interaction between birthweight and the *CASR* with regard to BMD among women, but not men. Our study group were some years past menopause and hence their BMD would reflect the peak bone mass attained and the rate of subsequent bone loss. It is possible that the interaction we describe may reflect an interaction between birthweight and bone loss. Although the data we present are cross-sectional, the cohort is being followed up, and we will be able to test for an interaction with bone loss rate shortly.

Our study shows, for the first time, evidence of an interaction between a polymorphism of the *CASR* gene and growth in early life in the determination of bone mass in later life in a UK female population. Further work is now indicated to replicate this finding.

ACKNOWLEDGMENT

We thank the men and women who participated in the study, the general practitioners who allowed access to their patients, and the nurses and radiology staff who administered the bone density measurements. Computing support was provided by Vanessa Cox.

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